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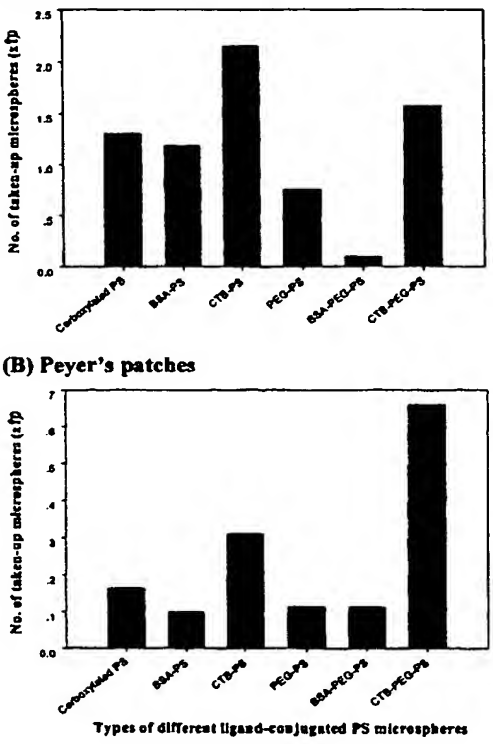
(54) Title: SURFACE MODIFIED MICROSPHERES WITH CHOLERA TOXIN B SUBUNIT

(57) Abstract: The present invention relates to microspheres whose surface is conjugated with cholera toxin B subunit (CTB), directly or indirectly via polymer spacer, which is useful for an orally administrable formulation of various biologically active substances due to the high uptake efficiency in intestine.



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SURFACE MODIFIED MICROSPHERES WITH CHOLERA TOXIN B SUBUNIT

FIELD OF THE INVENTION

The present invention relates to microspheres whose surface is modified by the conjugation of cholera toxin B subunit (CTB), particularly to microspheres conjugated with CTB directly ('CTB-microsphere') or indirectly via polymer spacer ('CTB-spacer-microsphere').

This invention also relates to processes for preparing the microspheres and to pharmaceutical compositions using the microspheres.

BACKGROUND

Oral administration is one of the attractive methods in delivering medicinal agents such as therapeutic drugs and vaccines. It has many advantages such as easy administration of medicinal agents, lack of pain, no need for trained personnel, and a cost effectiveness (Fasano, *Trends in Biotech.*, 16:152-157, 1998). However, many drugs are hard to be absorbed through animal intestinal epithelium. Moreover, orally administered protein and peptide drugs cannot be delivered to the absorptive site of intestine because

of proteolytic digestive enzymes and low pH in stomach. For these reasons, it would be desirable to develop the effective protein dosage form to deliver them to the systemic circulation or target organ through the enterocytes.

There are many reports that proteins in the forms of microspheres are effectively protected from enzymatic or acidic degradation. In addition to protection, the use of delivery system to target the drugs to the intestinal absorptive site is more desirable to enhance the uptake probability of drugs. Especially particulate systems can be taken up through mucosal membrane.

Recently, oral vaccine, one of the mucosal vaccines, has become a hot topic. The mucosal membranes of the body are the common sites of pathogenensis, and almost all viral, bacterial, or parasitic pathogenic organisms causing common infectious diseases of the gastrointestinal (GI), respiratory, and genitourinary tract enter or infect through a large surface area (about 400 m^2) of mucosal membranes (Mestecky *et al.*, *J. Control. Release*, 48:243-257, 1997).

The mucosa-associated lymphoid tissue (MALT) is the major site for antigen sampling and generation of specific effector cells and memory cells. One of the well-known examples of MALT is gut-associated lymphoid

tissue (GALT). It comprises the Peyer's patches, the mesenteric lymph nodes, and many of lymphoid cells scattered throughout the lamina propria and epithelium of the intestine. The PP are the sites to produce antigenic stimulation and their dome regions are covered with the follicle-associated epithelial cells (M cells) which actively transcytose luminal contents.

The M cell differs from adjacent absorptive enterocytes. It has microfold, short, irregular microvilli and is covered with the thin mucin-like molecules called the glycocalyx, whereas the apical surfaces of the absorptive enterocytes are formed by rigid, compactly packed microvilli and coated with thick glycocalyx which functions as a diffusion barrier and a degradative microenvironment. Many transport vesicles exist in the apical cytoplasm of the M cells and the basolateral membrane is invaginated to form the intraepithelial pocket (see FIG 1). It provides a pathway to endocytose and transport macromolecules, microorganisms, viruses, and microparticles. The transported molecules are processed by antigen-presenting accessory cells and lymphocytes, and stimulate the host's local immune responses. The local immune response is the mucosal immunity and dependent on transporting antigens through the M cells (Wolf and Bye, *Ann. Rev. Med.*, 35:95-112, 1984; Neutra *et al.*, *Ann. Rev. Immunol.*, 14:275-300, 1996). Therefore, the

transcytosis of particulated systems through the M cells is one of the possible routes of intestinal uptake of oral vaccines. However, it is still required that antigens in oral vaccines should be delivered to their uptake sites without loss of their activities, since most of antigens contain proteins, polypeptides, or polysaccharides.

There are many factors to determine the fates of orally administered microspheres. First of all, the size of microspheres influences the uptake through the PP M cells in the GI tract. Also depending on the size of microspheres, the distribution of taken-up microspheres is determined and both systemic and mucosal immunity can be induced concomitantly or selectively after the oral delivery. Smaller (up to 500 nm) microspheres are taken up efficiently than larger ones. A biodegradable and biocompatible polymer, poly D,L-lactide-co-glycolide (hereinafter, referred to as 'PLGA'), has been most frequently used in oral delivery. Both the uptake into the PP and the other mucosa-associated lymphoid tissue and the biological fate after uptake are determined by PLGA microsphere size (Eldridge, *J. Control Release*, 11:205-214, 1990; Mestecky *et al.*, *J. Control Release*, 28:131-141, 1994). PLGA microspheres larger than about 10 μm in diameter are known not to be translocated to the PP. Smaller microspheres than 10 μm in diameter are taken up into

the PP. Those ranged from 5 to 10 μm in diameter remain in the PP and those smaller than about 5 μm can migrate from the PP to the MLN, the spleen, and other deep tissues (liver, kidney, etc). And polystyrene (PS) microspheres in the size range of 0.05 to 1 μm migrated through the PP to the liver, the spleen, the blood and the bone marrow but 3 μm PS microspheres did not (Jani *et al., J. Pharm. Pharmacol.,* 41:809-812, 1989). These results of uptake and distribution suggest that microsphere size may be a determinant in the type of immune response elicited by antigen encapsulated within microspheres administered in the oral route. Antigens remaining in the PP induce a mucosal immune response and those translocated from the PP to the spleen induce both a systemic immune response and secrete secretory IgA.

The hydrophobicity of microspheres also affects the uptake into the PP. The relatively more hydrophobic polymers such as polystyrene, poly(methyl methacrylate) and poly(hydroxybutyrate), were readily absorbed. One exception is ethyl cellulose whose efficiency of absorption was found to correlate with the relative hydrophobicity of the surface of microspheres. The major determinant in the absorption of microspheres larger than 10 μm is the hydrophobicity of polymer. The uptake level of PS microspheres coated with hydrophilic surfactant poloxamer™ was low in the

small intestine region of the gut. It seemed that coated poloxamer™ blocked the uptake of microspheres in the small intestine (Florence, J. Control Release, 36:39-46, 1995). Negatively charged carboxylated PS microspheres were taken up to a less degree than non-ionic microspheres (Jani *et al.*, *J. Pharm. Pharmacol.*, 41:809-812, 1989).

The binding of microspheres to the M cells depends on the surface properties of both cells and microspheres. It is suggested that the surface modification may enhance the efficiency of the microsphere delivery vehicles. One approach is the conjugation of microsphere with molecules that have binding specificity for the M cells. The coating of microsphere with different proteins affected their uptake into the M cells. Smith *et al.* (Smith *et al.*, *Exp. Physiol.*, 80:735-743, 1995) reported that a binding selectivity of the protein coated to microspheres for the M cells had the order of bovine growth hormone < bovine serum albumin < human IgG in mice, and bovine growth hormone < bovine growth hormone - Ab in mice and rats. M cell-specific IgG and IgA could facilitate both the binding and the entry of microspheres into the M cells. The uptake of 1 μm PS microspheres attached with a specific monoclonal antibody for the rabbit M cells increased in the rabbit PP, whereas a non-specific monoclonal antibody had no

effect (Pappo *et al.*, *Immunology*, 73:277-280, 1991; Ermak and Giannasca, *Adv. Drug Del. Rev.*, 34:261-283, 1998). Lectins bound to the surfaces of microspheres induce the increased uptake into the absorptive cells as well as lymphoid tissues, but the lectin binding properties to the intestinal M cells among species are quite different, depending on the type and origin of bound lectins (Clark *et al.*, *Cell Tissue Res.*, 282:455-461, 1995; Jepson *et al.*, *J. Anat.*, 189:507-516, 1996). Recently, invasin protein, adhesion molecule of certain pathogenic bacteria such as *Yersinia*, *Shigella* and *Salmonella*, was used for the M cell targeting ligand (Clark *et al.*, *Infect. Immun.*, 66:1237-1243, 1998).

One of the strategies to enhance the immunogenecity of orally administered antigens was to couple them to cholera toxin (CT) or its non-toxic binding B subunit (hereinafter, referred to as 'CTB'). It was known as the adjuvant of mucosal immunity (Czerkinsky, *Infect. Immun.*, 57:1072-1077, 1989). The mutants of cholera toxin devoid of toxicity maintained a mucosal adjuvant activity (Douce *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:1644-1648, 1995). It was reported that CTB-conjugated liposome containing antigen induced effectiviely mucosal as well as systemic immune responses when administered in the oral route (Harokopakis *et al.*, *J. Immunol. Method*, 185:31-42, 1995; Harokopakis *et al.*, *Infect. Immunity*, 66:4299-

4304, 1998).

The binding specificity of CTB is responsible for its adjuvant activity of mucosal immunity. CTB can bind specifically to GM_1 ganglioside but not to mucins. The binding of CTB to glycolipid receptor is not specific to the M cells because GM_1 ganglioside is present in the apical membranes of all intestinal epithelial cells. However, as described above, the surface morphology of the M cells is different from that of absorptive cells. Therefore, CTB may bind to GM_1 ganglioside on the M cells more frequently than to that on absorptive cells, and the uptake efficiency of microspheres modified with CTB may depend on the surface topology of the microsphere and on microenvironment surrounding the microsphere.

Based upon this notion, we, the inventors of the present invention, have developed a series of microspheres whose surface is modified with CTB directly or via polymer spacer. We confirmed that oral administration of the microspheres is followed by efficient uptake into intestine cells, especially into PP, thus verifying that the microspheres may be employed as an effective delivery system for the oral vaccines etc.

SUMMARY OF THE INVENTION

It is an object of this invention to provide CTB-modified microspheres showing higher efficiency of uptake into small intestine.

It is a further object of this invention to provide microspheres that are effectively taken up into PP and may be employed as a delivery system for the oral vaccines etc.

Further objects and advantages of the present invention will appear hereinafter.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides microspheres whose surface is modified with CTB or variants thereof.

This invention also provides microspheres whose surface is conjugated with CTB or a variant thereof via polymer spacer.

In addition, this invention provides orally administerable, pharmaceutical compositions or vaccines in which said microspheres are used as pharmaceutical vehicles.

Also this invention provides processes for preparing said microspheres.

Further features of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts M cells ('M') overlying a PP and absorptive enterocytes,

FIG. 2 depicts the six types of different ligand-conjugated polystyrene (PS) microspheres,

FIG. 3 schematically depicts the conjugation of *t-Boc*-NH-PEG_{3400}-NH_2 to carboxylated PS microspheres,

FIG. 4 schematically depicts the modification of PEG_{3400}-conjugated PS microsphere with sulfo-LC-SPDP,

FIG. 5 schematically depicts the modification of CTB with sulfo-SMPB,

FIG. 6 schematically depicts the conjugation between CTB-MPB and TPH-PEG_{3400}-conjugated PS microsphere,

FIG. 7 is standard curve of the number of 1 μm FITC-, carboxylated PS microspheres versus the intensity of fluorescence,

FIG. 8 is scanning electron microscopy photographs of 1 μm FITC-, PS microspheres,

top: the microspheres before TFA treatment;

bottom: the microspheres after TFA treatment,

FIG. 9a and 9b depict the effect of CTB:suflo-SMPB molar ratio and incubation time on the binding ability of CTB-MPB to the GM_1 ganglioside,

circle: the molar ratio 1:10;

inverted triangle: the molar ratio 1:50;

rectangle: the molar ratio 1:100;

diamond: unmodified CTB (No sulfo-SMPB),

FIG. 10 depicts the SDS-PAGE analysis of BSA-MPB (Bovine Serum Albumin modified with sulfo-SMPB),

lane M: molecular marker (in kilodalton);

lane 1: unmodified BSA;

lane 2: mixture of unmodified BSA and BSA-MPB;

lane 3: BSA-MPB,

FIG. 11 depicts the SDS-PAGE analysis of CTB-MPB (Cholera Toxin B subunit modified with sulfo-SMPB),

lane M: molecular marker (in kilodalton);

lane 1: unmodified CTB;

lane 2: mixture of unmodified CTB and CTB-MPB;

lane 3: CTB-MPB,

FIG. 12 depicts the uptake efficiency of six types of microspheres in the small intestine,

top: uptake efficiency into absorptive cells;

bottom: uptake efficiency into PP.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides microspheres whose surface is modified with CTB or variants thereof.

Said 'CTB (Cholera Toxin B subunit)' is exemplified by a polypeptide which is described by GenBank Accession Number BAA06289. Said 'variants thereof' refers to proteins that can be prepared from said CTB by substitution, deletion or insertion of at least one amino acid residue in CTB and show 50% or more of binding activity to GM_1 ganglioside when compared with that of the CTB. Several references describe CTB and variants thereof, including Czerkinsky, *Infect. Immun.*, 57:1072-1077, 1995; Douce *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:1644-1648, 1995; Harokopakis *et al.*, *J. Immunol. Method*, 185:31-42, 1995; and Harokopakis *et al.*, *Infect. Immun.*, 66:4299-4304, 1998.

Said 'microsphere' refers to a spheral particle with micrometer scale in diameter. It may be composed of various materials such as starch, dextran, puluran, chitosan, alginate, cellulose, gelatin, albumin, polyvinyl alcohol, polymethylmethacrylate, polystyrene, polyimidocarbonate, polyvinylpyrrolidone, poly(DL-lactide-co-glycolide), polyorthoesters, polyanhydride, polyphosphazene, and so on. More preferable materials includes starch, dextran, puluran, chitosan, alginate, cellulose, gelatin, albumin, poly(DL-lactide-co-glycolide), polyorthoesters, polyanhydride, and polyphosphazene, since these biodegradable polymers can be decomposed after the active substance is released from the microspheres.

The diameter of the microspheres is preferably about 0.5 ~ 5 μm, and preferred embodiments of this invention employed 1 μm microspheres. It has been reported that microspheres with 5 μm in diameter or more cannot be absorbed through intestine, and those with 0.5 μm or less in diameter do not effectively have access to its receptors on the cell surface.

The microspheres provided by this invention may be conjugated with CTB directly (i.e. 'CTB-microsphere') or indirectly via polymer spacer (i.e. 'CTB-spacer-microsphere'). The region of polymer spacer should be sufficient spacing and flexibility between the microsphere and CTB so that the CTB is available to interact with the target molecule, GM1 ganglioside. In addition, whether the microspheres is conjugated with CTB directly or indirectly, the microsphere may be conjugated with CTB covalently or noncovalently.

Precisely, covalent binding can be achieved through the use of a suitable linking region or group and non-covalent binding can be achieved by utilizing strong ionic interaction, hydrophobic interactions, Van der Waals forced, and the like. Covalent binding between microsphere or polymer-spacer conjugated microsphere ('spacer-microsphere') and CTB will be preferably achieved using bifunctional compound having a reactive group at one end which capable of binding to microsphere or spacer-microsphere and a second reactive

group at the other end which is capable of binding to CTB.

In a preferred embodiment, 1-ethyl-1,3-(3-dimethyl-aminopropyl)carbodiimide (EDC) is employed for direct conjugation between microsphere and CTB, and succinimidyl compound is employed for conjugation between spacer-microsphere and CTB.

Since polymer spacer may increase the accessibility to the PP(Payer's Patches), CTB-spacer-microsphere may be taken up dominantly into the PP and is more preferable for oral vaccines and the like. Polymer spacer used for conjugation of CTB with microsphere can be selected from polyethyleneglycol, polyethyleneimine, polyethyleneoxide, jeffamine, polyprophyleneoxide, polyacrylic acid, polymethacrylic acid or poly-L-lysine.

The preferred embidiments of this invention employed polyetyleneglycol.

FIG 2 shows the six types of different ligand-conjugated 1 μm polystyrene (PS) microspheres.

Any type of PEG may be used in the PEG spacer, although PEG with molecular weight range of 3,000 ~ 4,000 is more preferable. Preferred embodiments of this invention employed PEG with MW 3,400 (that is, PEG_{3400}). If molecular weight of PEG is too large for the size of microsphere, the PEG molecule will significantly affect the surface property or the size of the microsphere.

In contrast, too small PEG spacer will not function as a spacer for CTB. In conclusion, PEG with molecular weight of 3,000 ~ 4,000 is preferable.

In a preferred embodiment, employed is PEG molecule with two amino groups at both ends, one of which is protected by *t-Boc* group (*t-Boc*-NH-PEG_{3400}-NH_2 spacer). This type of PEG spacer is more preferable than PEG spacer without any protective group, since the protective group can prevent the microspheres from being conjugated each other.

The CTB-PEG-PS microsphere of this invention may be prepared according to following reactions:

1) conjugation of PEG to microspheres (see FIG 3),
2) conjugation of sulfo-LC-SPDP (sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido]hexanoate) to the PEG-microspheres, producing TPH-PEG-microsphere (TPH: thiopropionamidohexanoate; see FIG 4),
3) modification of CTB with sulfo-SMPB (sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate) (see FIG 5), and
4) conjugation of the modified CTB to the TPH-PEG-microspheres (see FIG 6)

In the reaction 3), molar ratio of CTB to sulfo-SMPB is preferably about 1:10, and the reaction time is desirably 2 hours or less, since CTB modified under such condition shows the most similar level of binding activity, compared with unmodified CTB.

Fluorescents such as fluoroscein isothiocynate (FITC) may be conjugated to the surface of the microspheres of this invention. The fluorescents are useful for the detection of the microspheres, and microspheres prepared in Examples and Comparative Examples contain the FITC.

This invention also provides orally administerable, pharmaceutical compositions or vaccines in which said microspheres are used as pharmaceutical vehicles.

In preferred embodiments, the microspheres of this invention show remarkable uptake efficiencies into the absorptive enterocytes and the PP (see FIG 12 and Table 2). Thus, it is apparent that the microsphere of this invention can be used as a orally administrable vaccine, a orally administrable pharmaceutical composition, and so on.

An active substance in said vaccine or said pharmaceutical composition preferably includes a substance that is subject to inactivation and degradation when orally administered, and a substance that is poorly taken up into small intestine. These substances are referred to as a 'biologically active substances', which is exemplified by macromolecules such as proteins, polypeptides, polysaccharides, lipids, and nucleic acids; by small therapeutic agents that is poorly taken up into small intestine, and by various

types of antigen. Said 'antigen' refers to a material that is employed as a active substance for conventional vaccines. The antigen may elicit systemic or local immune responses in an animal or in a human.

Positioned either in the internal space or on the surface of the microspheres, the biologically active substance may be prevented from being degraded in oral administration. In addition, more than one biologically active substance may be applied in combination with other biologically active substance.

The effective dosage of the pharmaceutical composition or vaccine depends on the nature of a biologically active substance it contains.

In another aspect of this invention, processes also are provided for preparing the microspheres of this invention.

The microsphere that is directly conjugated with CTB may be prepared according to the following steps of:

1) activating carboxyl groups on microspheres with 1-ethyl-1,3-(3-dimethyl-aminopropyl)carbodiimide (EDC), and
2) conjugating CTB or a variant thereof to the activated carboxyl groups.

In addition, the microsphere that is indirectly conjugated with CTB via PEG spacer may be prepared

according to the following steps of:

1) preparing PEG-microspheres by conjugating PEG to microspheres,

2) preparing TPH-PEG-microspheres by conjugating sulfo-LC-SPDP to the PEG-microspheres,

3) modifying CTB or a variant thereof with sulfo-SMPB, and

4) preparing CTB-PEG-microspheres by conjugating the modified CTB or a variant thereof to the TPH-PEG-microspheres.

In the step 3), molar ratio of CTB to sulfo-SMPB is preferably about 1:10, and the reaction time is desirably 2 hours or less.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Preparation of CTB-PS microsphere

The conjugation between FITC-, carboxylated polystyrene(PS) microsphere and protein was

accomplished by carbodiimide coupling method.

Particularly, the monodisperse carboxylated PS microspheres (nominally 1 μm in diameter) with covalently linked fluorescein isocyanate (FITC) were purchased in suspension from Polyscience Inc. (Warrington, PA, USA). The properties of PS microspheres are listed in Table 1. 50 μl of FITC-, carboxylated PS microspheres in stock solution of 2.7% (w/v) suspension were washed with 0.1 M MES solution (2-[morpholino]ethanesulfonic acid, pH 4.5). Carboxyl groups of 50 μl of FITC-, carboxylated PS microspheres were activated with 100 μl of 0.2 M EDC [1-ethyl-1,3-(3-dimethyl-aminopropyl)carbodiimide; Pierce Chemical Co., Rockford, USA] by incubating at room temperature at pH 4.5 for 2 hours with shaking gently. Then this reaction mixture was centrifuged at 20,000g for 30 minutes and the supernant was discarded. 100 μg of CTB (List Biological Inc., USA) dissolved in 0.1 M MES solution at 1 mg/ml was added to FITC-, PS microspheres activated with EDC and incubated at room temperature for 2 hours with shaking gently. After the reaction, this reaction mixture was centrifuged at 20,000g for 30 minutes, and the supernatant was transferred. Directly protein-conjugated PS microspheres were washed with phosphate buffered saline (PBS; 0.20 g/l KCl, 0.20 g/l KH_2PO_4, 8.00 g/l NaCl, $Na_2HPO_4 \cdot 12H_2O$, pH 7.4) in order to remove traces of unreacted protein.

Table 1

Number of microspheres per gram	Number of carboxyl groups per gram	Number of carboxyl group per microsphere
1.97×10^{12}	$3.31 \times 10^{19} \pm 0.3 \times 10^{11}$	$1.82 \times 10^{7} \pm 0.17 \times 10^{7}$

Example 2: Preparation of CTB-PEG-PS microspheres

(2-1) Conjugation of FITC-, carboxylated PS microspheres with *t-Boc*-NH-PEG_{3400}-NH_2 spacer

The conjugation between FITC-, carboxylated PS microspheres and *t-Boc*-NH-PEG_{3400}-NH_2 spacer was also accomplished by carbodiimide coupling method (see FIG 3). Carboxyl groups of FITC-, carboxylated PS microspheres were activated with EDC and directly conjugated with *t-Boc*-NH-PEG_{3400}-NH_2 spacer, which is polyethylene glycol with molecular weight 3400 that there were two amino groups at both ends and that one of them was protected by *t-Boc* group.

Particularly, 50 μl of FITC-, carboxylated PS microspheres in stock solution of 2.7% (w/v) suspension was washed with 0.1 M MES solution and then suspended in 100 μl of 0.1 M MES. 100 μl of 0.2 M EDC and *t-Boc*-NH-PEG_{3400}-NH_2 spacer (Shearwater Polymers, Inc., USA) dissolved in 0.1 M MES at *t-Boc*-NH-PEG_{3400}-NH_2 spacer : carboxyl groups in microspheres molar (3:100) was added to this suspended solution and incubated at room temperature for 24 hours with shaking gently. Then the

reaction mixture was centrifuged at 20,000g for 30 minutes, and the supernatant was removed. FITC-, PEG_{3400}-conjugated PS microspheres were washed with PBS. Trifluoroacetic acid (TFA, Janssen Chemica) was treated to FITC-, PEG_{3400}-conjugated PS microspheres for 1 hour to remove *t-Boc* group. To remove TFA, FITC-, PEG_{3400}-conjugated PS microspheres were dried in vacuum. Dried FITC-, PEG_{3400}-conjugated PS microspheres were washed several times with PBS solution at pH 7.4.

(2-2) Conjugation of FITC-, PEG_{3400}-conjugated PS microspheres with sulfo-LC-SPDP

Modification of thiopropionamidohexanoate (TPH)-PEG_{3400}-conjugated PS microspheres was outlined in FIG 4. In this scheme, functional amine groups of FITC-, PEG_{3400}-conjugated PS microspheres were changed to reactive sulfhydryl groups.

Particularly, functional amine groups of the FITC-, PEG_{3400}-conjugated PS microspheres prepared in Example (2-1) were suspended in 200 μl of PBS. Excess amount of 50 μl of 10 mM sulfo-LC-SPDP (sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido]hexanoate; Pierce Chemical Co., USA) was added and incubated at room temperature for 1 hour with shaking. Then the reaction mixture was centrifuged at 20,000g for 30 minutes and the supernatant was removed. FITC-, PEG_{3400}-conjugated PS microspheres reacted with sulfo-LC-SPDP were washed

and resuspended in 200 μl of PBS. 100 μl of 25 mM DTT (Boehringer Mannheim GmbH, Germany) in PBS was treated and incubated for 1 hour. The reaction mixture was centrifuged at 20,000g for 30 minutes. The supernatant was discarded. The DTT-treated microspheres were washed with PBS to remove DTT.

(2-3) Protein modification with sulfo-SMPB

In order to conjugate CTB protein (List Biological Inc., USA) to the TPH-PEG-PS microspheres prepared in Example (2-2), CTB protein was modified with sulfo-SMPB (sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate). Modification of CTB-4-(*p*-maleimidophenyl)butyrate (CTB-MPB) or bovine serum albumin-4-(p-maleimidophenyl) butyrate (BSA-MPB; prepared in Comparative Example 2) was outlined in FIG 5. In brief, the functional amine groups of CTB or BSA were changed to maleimide groups reactive to sulfhydryl groups of TPH-PEG_{3400}-conjugated PS microspheres using the water soluble amine-reactive reagent sulfo-SMPB (Pierce Chemical Co., USA).

In detail, 100 μl of proteins dissolved in PBS at pH 7.0 at 1 mg/ml were incubated with sulfo-SMPB at various CTB:sulfo-SMPB molar ratios (1:10, 1:50, 1:100) at 4°C for various incubation time (2 hours, 4 hours, 8 hours, and 24 hours). The reaction mixture was centrifuged at 14,000g for 50 minutes at 4°C to remove unreacted sulfo-SMPB by using Amicon Microcon

(molecular weight cut-off: 10,000).

(2-4) Conjugation of CTB-MPB to TPH-PEG_{3400}-conjugated PS microspheres

Conjugation of CTB-MPB to TPH-PEG_{3400}-conjugated PS microspheres was accomplished by incubating the modified protein with a suspension of TPH-PEG_{3400}-conjugated PS microspheres. The reaction scheme was outlined in FIG 6.

Particularly, 100 μg of the modified protein was added to the reactive sulfohydryl bearing TPH-PEG_{3400}-conjugated PS microspheres and incubated at room temperature for 30 minutes with shaking unreacted modified protein and washed several times with PBS.

Comparative Example 1: Preparation of BSA-PS microspheres

All the procedures were conducted according to Example 1, except that CTB was replaced by BSA (Sigma, USA).

Comparative Example 2: Preparation of BSA-PEG-PS microspheres

All the procedures were conducted according to

Example 1, except that CTB was replaced by BSA (Sigma, USA).

Experiment 1: Determination of the degree of BSA or CTB conjugation to PS microspheres

Bradford assay was used to determine the degree of protein conjugation to the surface of the microspheres which were prepared in Example 1 and Comparative Example 1. Unreacted protein in supernatant was quantitated using a Bradford protein assay kit (BioRad Laboratories, USA) as described by the manufacturer. The protein solution was normally assayed in duplicate.

In detail, a set of protein standards was prepraed using PBS as a diluent. The solutions of 160 μl each of standard, blank, and samples were pipetted into microtiter plate wells and 40 μl of dye reagent concentrate was added. The sample and the reagent were mixed thoroughly. The mixture was incubated at room temperature for at least 5 minutes, and then absorbance was measured at 595 nm using a microplate reader (Molecular Devices Corp, Sunnyvale, USA).

In case of BSA conjugation, about 40% of initial amount of BSA before the conjugation reaction was reacted with carboxyl groups of FITC-, PS microspheres (about 15.75 μg BSA per 1 × 10^9 microspheres). In case

of CTB conjugation, about 70% of initial amount of CTB before the conjugation reaction was successfully conjugated with carboxyl groups of FITC-, PS microspheres (about 27.56 μg CTB per 1 × 10^5 microspheres). This represents that about 1.57% of carboxyl groups on the surface of microspheres were conjugated with CTB.

Experiment 2: Observation of PEG-PS microspheres through SEM

TFA was used to prepare PEG-PS microspheres in Example (2-1). Since TFA is a strong acid and could change the surface morphology of FITC-, PEG_{3400}-PS microspheres, the surface morphology was observed by using scanning electron microscope (SEM; S-2460N, Hitachi Ltd., Japan). As shown in FIG 8, the surface has not changed by TFA treatment.

Experiment 3: Determination of binding activity of CTB-MPB to GM_1-ganglioside

To confirm binding capacity of CTB-MPB prepared in Example (2-3), ELISA (Enzyme-linked immunosorbent assay) was carried out.

Particularly, polystyrene microtiter plates (Nunc,

Denmark) were coated with 100 μl of 1 μg/ml GM_1 monosialoganglioside (Sigma Chemical Co., USA) or with 100 μl of 1 mg/ml BSA in bicarbonate coating buffer (40 mM Na_2CO_3, 60 mM $NaHCO_3$, pH 9.6) each well. After an overnight adsorption at 4°C, the contents of the plates were washed six times with PBS containing 0.05% Tween 20® (PBST). The wells were filled with PBST containing 5% skim milk and incubated at room temperature for 1 hour. The contents were discarded and the wells were washed six times with PBST. Samples were diluted serially by 2-fold from 1 μg/ml, and 100 μl per well was added. Following the incubation at room temperature for 1 hour, the contents were discarded and the wells were washed six times with PBST. To rule out the nonspecific bindings of CTB-MPB, the binding activity of CTB-MPB in the BSA-coated wells was tested as a control. Rabbit anti-CTB antibody obtained from hyperimmunized rabbit sera was diluted 1,000 times in PBST containing 1% skim milk, and 100 μl per well was added and incubated at room temperature for 1 hour. After washing with PBST, the plates were treated with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Organon Tekmika Corp., USA) diluted 5,000 times in PBST containing 1% skim milk at room temperature for 1 hour. The contents were discarded and the wells were washed six times with PBST. All reagents were used in a standard volume of 100 μl.

Stable peroxidase substrate buffer (Pierce Chemical Co., USA) containing 1 mg/ml OPD (o-phenylenediamine dihydrochlorite; Pierce Chemical Co., USA) was used to develop color. The reaction was terminated by adding 100 μl of 2 N H_2SO_4 and the optical density (O.D.) at 492 nm was measured by using a microplate reader (Molecular Devices Corp., USA). The samples were assayed in duplicates and a pair of blank well was included on each plate.

The modification of CTB with sulfo-SMPB influenced the binding activity to GM_1 ganglioside, as shown in FIG 9. ELISA was conducted after we carried out protein modification reactions at various ratios of CTB to sulfo-SMPB and for various reaction times, as described in Example (2-3). In result, the binding activity of CTB modified with sulfo-SMPB at a 1:10 molar ratio for 2-hour reaction time to GM_1 ganglioside was similar to that of unmodified CTB when compared with that of CTB-MPB at different molar ratio for different reaction time. The reaction time had slight influence on binding activity, whereas the molar ratio of sulfo-SMPB to CTB influenced the binding activity of CTB-MPB. Higher degree of the modification with sulfo-SMPB decreased the binding activity of CTB to GM_1 ganglioside.

Experiment 4: SDS-PAGE analysis of BSA-MPB and CTB-MPB

To confirm the protein modification in Example (2-3) and Comparative Example 2, the modified protein was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Maniatis et al. (Maniatis et al., Molecular Cloning: A Laboratory Manual, Second Edition, 1989, Cold Spring Harbor Laboratory Press, USA).

FIG 10 represents the 15% SDS-PAGE of BSA-MPB which was prepared from the reaction with 1:10 BSA-to-sulfo-SMPB molar ratio for 2 hours, whereas FIG 11 shows the 15% SDS-PAGE of CTB-MPB from the reaction with the same condition. Since both BSA-MPB and CTB-MPB had a different band pattern from unmodified BSA or CTB, it was verified that BSA and CTB were modified with sulfo-SMPB successfully.

Experiment 5: Determination of the degree of BSA or CTB conjugation to PEG-PS microspheres

As described in Experiment 1, Bradford assay was performed to determine the degree of protein conjugation to the surface of the microspheres which were prepared in Example (2-4) and Comparative Example 2.

In case of BSA-PEG_{3400}-PS microspheres, about 17.73 μg BSA-MPB per 1 × 10^9 microspheres was conjugated. In case of CTB-PEG_{3400}-PS microspheres, about 22.8 μg CTB-MPB per 1 × 10^9 microspheres was successfully conjugated. This represents that about 1.3% of carboxyl groups on the surface of microspheres were conjugated with CTB-MPB.

Experiment 6: Determination of binding activity of CTB-PEG-PS microsphere to GM_1-ganglioside

To confirm binding capacity of CTB-MPB prepared in Example (2-3), ELISA (Enzyme-linked immunosorbent assay) was carried out as described in Experiment 3.

In result, it was verified that conjugated CTB to PEG_{3400}-PS microspheres retained the binding activity to GM_1 ganglioside.

Experiment 7: Uptake of microspheres in vivo

14-week-old female Balb/c mice (purchased from Daehan Laboratory Animal Research Center Co., Korea) were used for the microsphere uptake experiments. Each mouse in six groups, fasted for 12 hours prior to oral feeding, was orally administered with a single gavage of 200 μl of six types of 1 μm FITC-, PS microspheres

of 1×10^9 suspended in PBS at pH 7.4 with 3% Na_2CO_3 using a blunt-tipped feeding needle. Each group had 5 mice (n=5). Mice were sacrificed 3 hours after administration with an overdose of anesthetic ether, and tissues were analyzed for the microsphere uptake. The MLN and the spleen were sampled prior to excising the small intestine to prevent contamination of the samples. Then the PP regions and the absorptive cells in the small intestine were dissected. All dissected tissues were washed with PBS to remove unabsorbed FITC-, PS microspheres and weighed. Tissue samples were dissolved by 3 ml of tissue dissolution solution (1% Triton X-100, 1% KOH) and heated to 60°C for 72 hours. Dissolved samples were centrifuged at 20,000g for 1 hour and the supernatant was pipetted off. 100 μl of PBS was added to the sample pellet, followed by resuspension of pellet. The number of taken-up microspheres was manually determined by using a fluorescence microscope (Nickon, Japan) equipped with optical filters for detecting fluorescein (Ebel, *Pharm. Res.*, 7:848-851, 1990; Hodge *et al.*, *Dig. Dis. Sci.*, 40:967-975, 1995).

To adjust the number of 1×10^9 1 μm FITC-, PS microspheres for a single gavage, multifrequency phase Fluorometer (ISS Inc., USA) was used. Serially diluted 1 μm FITC-, PS microspheres were treated with dimethylformamide and then intensity of extracted FITC

was measured by using fluorescence detector at 458 nm excitation wavelentgh and 540 nm emission wavelength. A standard curve of the number of 1 μm FITC-, PS microspheres to the intensity of FITC was constructed (see FIG 7).

Unpaired, two-tailed Students' *t*-tests were used to compare the mean numbers of FITC-, PS microspheres taken up into each tissue sample between groups of the mice. The value were considered statistically significant with p value < 0.05.

FIG 12 shows that the number of microspheres taken up into each part of the GI tract 3 hours after oral administration of six types of different ligand-conjugated 1 μm PS microspheres. The numbers of microspheres taken up into the 1 mg of tissue are summarized in Table 2. All types of microspheres were apparently taken up into the PP and the absorptive cells of the GI tract. Two types of CTB-conjugated PS microspheres (CTB-PS microspheres and CTB-PEG_{3400}-PS microspheres) were taken up into both the PP and the intestinal absorptive cells more than the others (carboxylated PS microspheres, BSA-PS microspheres, PEG_{3400}-PS microspheres, and BSA-PEG_{3400}-PS microspheres).

In the PP, CTB-PEG_{3400}-PS microspheres were taken up more than other types of different ligand-conjugated microspheres. In particular, the mean number of the uptake of CTB-PEG_{3400}-PS microspheres was more two times

than that of CTB-PS microspheres. Frey *et al.* (*J. Exp. Med.*, 184:1045-1059, 1995) reported CTB coated to 1 μm nonionic PS microspheres did not enhance the uptake into the PP than that of uncoated PS microspheres. Our result indicates that the accessibility of the CTB binding to GM_1 ganglioside in the apical membrane of the M cells increased by using spacer. Microspheres taken-up were not detected in the MLN and spleen 3 hours after oral administration in any of six different groups.

Table 2.

Tissue	Number of uptaken microspheres					
	Carboxylated PS	BSA-PS	CTB-PS	PEG-PS	BSA-PEG-PS	CTB-PEG-PS
PP	21.4 ± 3.2	14.6 ± 6.1	58.8 ± 27.1	21.0 ± 9.9	19.7 ± 4.6	105.5 ± 52.1
Absorptive cells	125.7 ± 11.2	126.9 ± 42.7	201.8 ± 142.2	86.4 ± 51.4	9.8 ± 5.4	155.0 ± 90.1
MLN	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Spleen	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Values are the mean numbers of uptaken microspheres ± standard error from five mice.

N.D. is for Not Detected.

INDUSTRIAL APPLICABILITY

The CTB-conjugated microspheres of this invention are efficiently taken up into both the PP and the absorptive cells of intestine, and especially the CTB-conjugated microsphere via PEG spacer is more

efficiently absorbed into the PP than any other microspheres. Therefore, CTB-conjugated microsphere or CTB-PEG-linked microsphere of this invention can be useful for an orally administrable formulation which contains various biologically active substances such as antigens and the like.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is Claimed is

1. A microsphere whose surface is modified with cholera toxin B subunit (CTB) or a variant thereof.

2. The microsphere of Claim 1, wherein the cholera toxin B subunit is conjugated to the microsphere surface via polymer spacer.

3. The microsphere of Claim 1, wherein polymer spacer is selected from polyethyleneglycol, polyethyleneimine, polyethyleneoxide, jeffamine, polyprophyleneoxide, polyacrylic acid, polymethacrylic acid or poly-L-lysine.

4. The microsphere of Claim 3, wherein the polyethylene glycol (PEG) spacer is 3,000 ~ 4,000 in molecular weight.

5. The microsphere of Claim 1, which, either in the internal space or on the surface, contains at least one biologically active substance.

6. The microsphere of Claim 5 wherein the biologically active substance is selected from the group comprising proteins, polypeptides,

polysaccharides, lipids, nucleic acids, small therapeutic agents, and antigens.

7. The microspheres of Claim 5, which, either in the internal space or on the surface, contains at least one species of a substance selected from the group comprising whole cell antigens, inactivated pathogenic organisms, attenuated live pathogenic organisms, recombinant or synthetic toxoids, haptens, tolerogens, and autoantigens.

8. The microsphere of Claim 1, which is 0.5 ~ 5 μm in diameter.

9. The microsphere of Claim 1 wherein the material of the microsphere is selected from the group comprising starch, dextran, puluran, chitosan, alginate, cellulose, gelatin, albumin, polyvinyl alcohol, polymethylmethacrylate, polystyrene, polyimidocarbonate, polyvinylpyrrolidone, poly(DL-lactide-co-glycolide), polyorthoesters, polyanhydride, and polyphosphazene.

10. An orally administerable, pharmaceutical composition in which the microsphere of Claim 1 is used as a pharmaceutical vehicle and which contains the biologically active substance

described in Claim 6.

11. An orally administerable vaccine which contains the microsphere of Claim 7.

12. A process for preparing the microsphere of Claim 1 comprising the steps of:

1) activating carboxyl groups on microspheres with 1-ethyl-1,3-(3-dimethyl-aminopropyl)carbodiimide (EDC), and

2) conjugating CTB or a variant thereof to the activated carboxyl groups.

13. A process for preparing the microsphere of Claim 4 comprising the steps of:

1) preparing PEG-microspheres by conjugating PEG to microspheres,

2) preparing TPH-PEG-microspheres by conjugating sulfo-LC-SPDP to the PEG-microspheres,

3) modifying CTB or a variant thereof with sulfo-SMPB, and

4) preparing CTB-PEG-microspheres by conjugating the modified CTB or a variant thereof to the TPH-PEG-microspheres.

14. The process of Claim 12, wherein the molar ratio of CTB to sulfo-SMPB is 1:10 and the reaction time

is 2 hours or less in step 3).

FIG. 1

FIG. 2

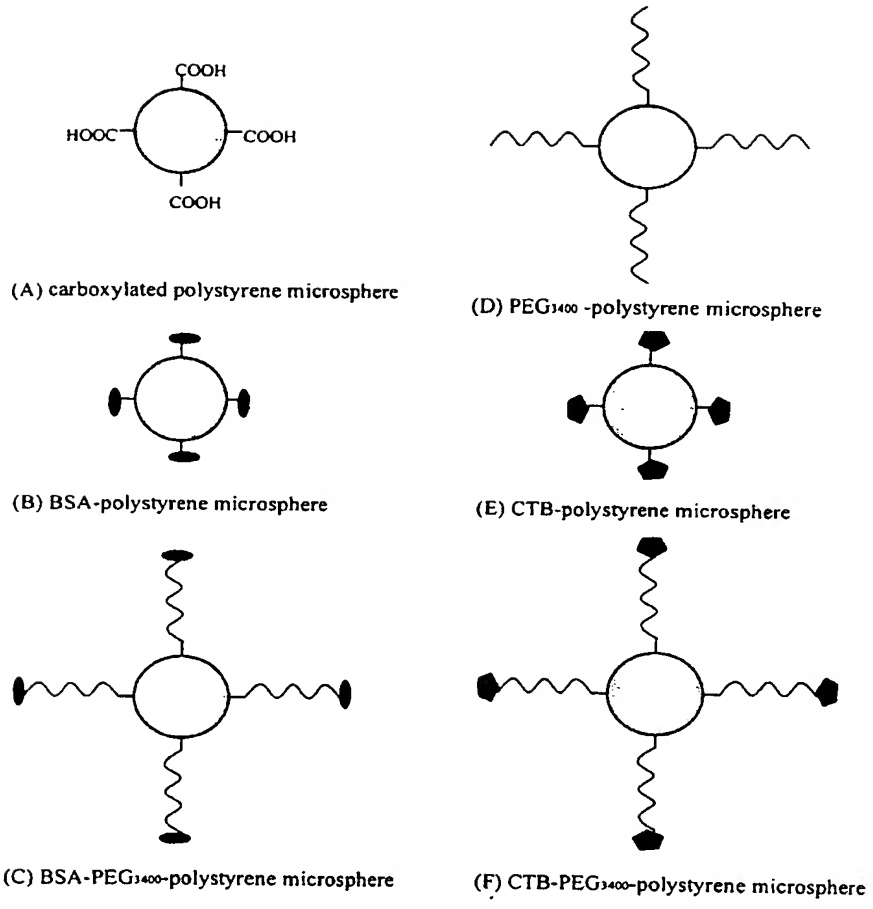
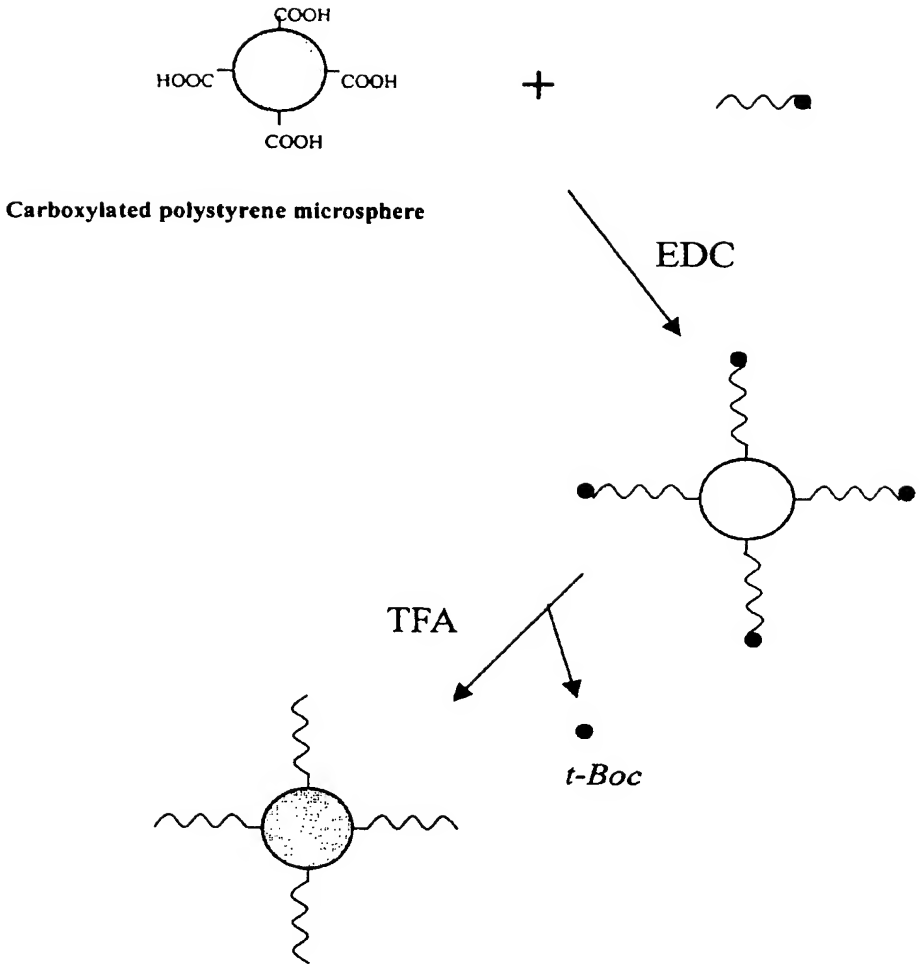

COOH
HOOC
COOH
COOH
(A) carboxylated polystyrene microsphere
(D) PEG3400 -polystyrene microsphere
(B) BSA-polystyrene microsphere
(E) CTB-polystyrene microsphere
(C) BSA-PEG3400-polystyrene microsphere
(F) CTB-PEG3400-polystyrene microsphere
: Bovine serum albumin (BSA)
: Cholera toxin B subunit (CTB)
: Polyethylene glycol (PEG3400)

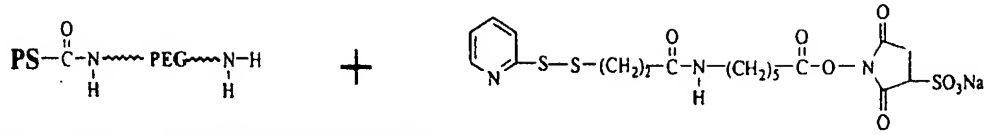
FIG. 3



Carboxylated polystyrene microsphere

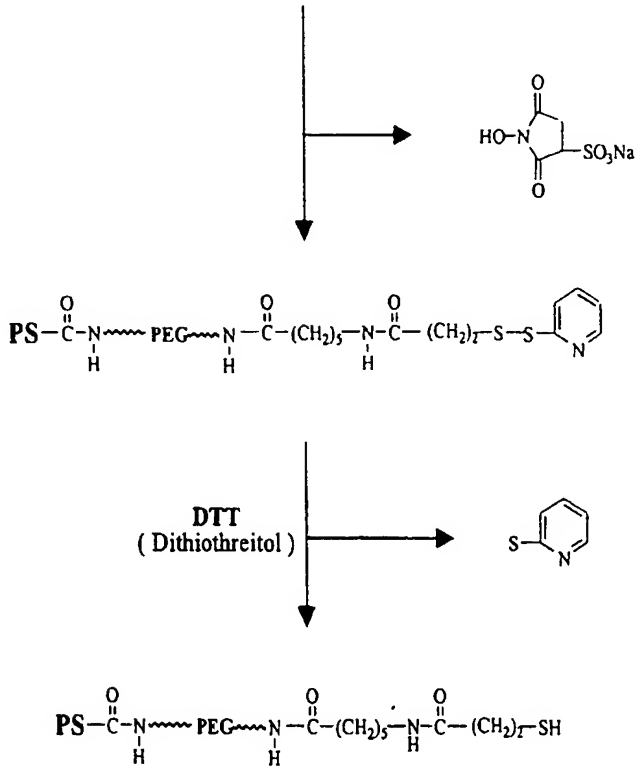
PEG_{3400}-conjugated polystyrene microsphere

FIG. 4



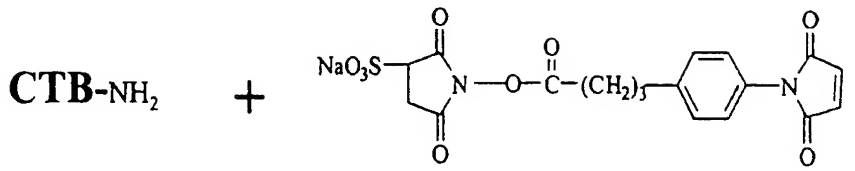
PEG_{3400}-conjugated polystyrene microsphere

Sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido]hexanoate



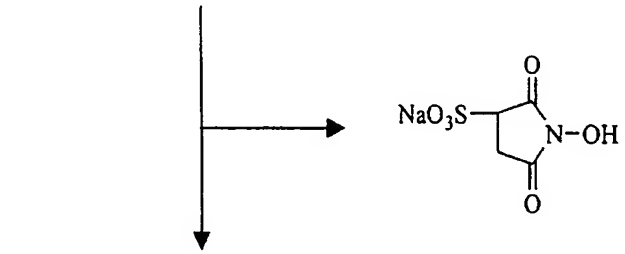
TPH-PEG_{3400}-PS (Thiopropionamidohexanoate-PEG_{3400}-conjugated polystyrene microsphere)

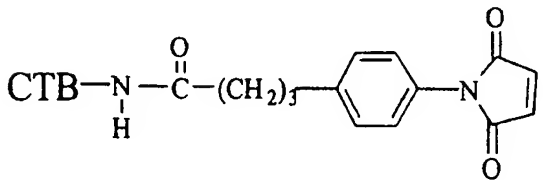
FIG. 5



Cholera toxin B subunit

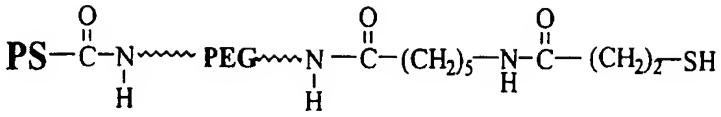
Sulfo-SMPB (sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate)





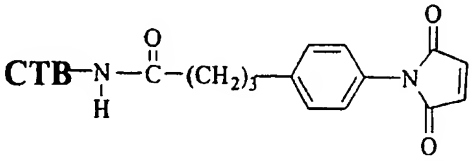
CTB-MPB (Cholera toxin B subunit-4-(*p*-maleimidophenyl)butyrate)

FIG. 6

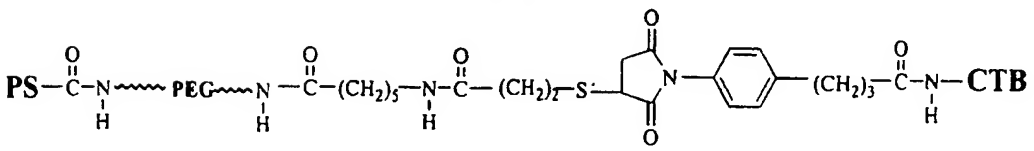


TPH-PEG_{3400}-PS (Thiopropionamidohexanoate-PEG_{3400}-conjugated polystyrene microsphere)

+



CTB-MPB (Cholera toxin B subunit-4-(*p*-maleimidophenyl)butyrate)



CTB-PEG_{3400} -PS (CTB-PEG_{3400} conjugated polystyrene microsphere)

FIG. 7

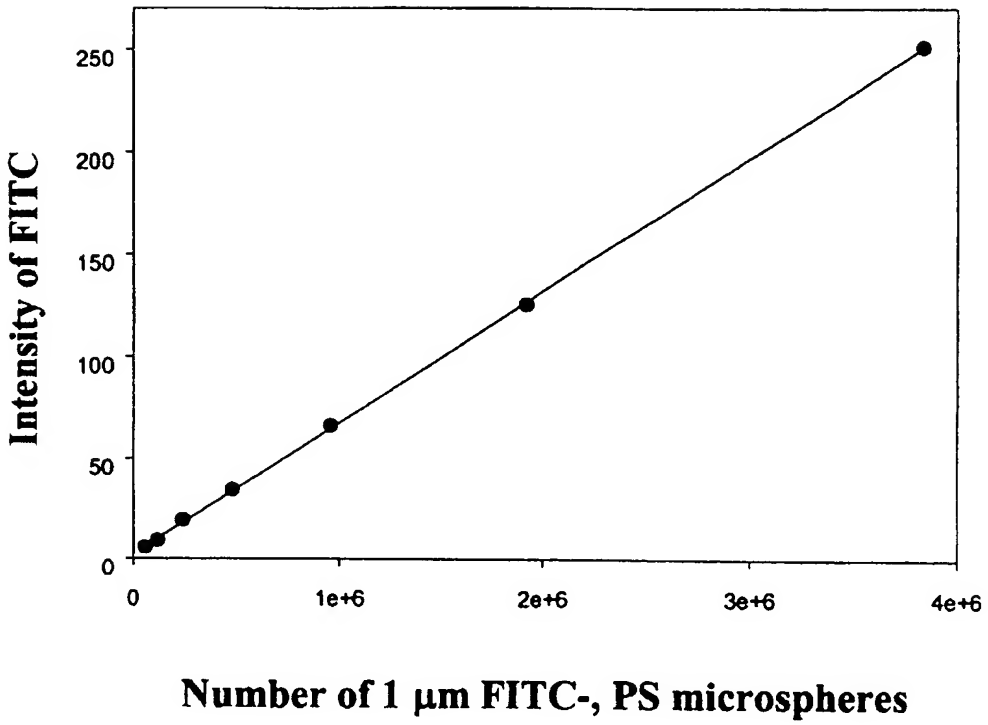
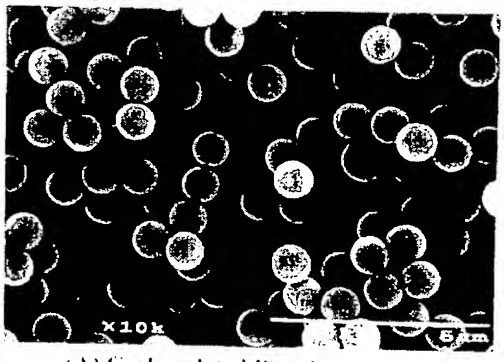
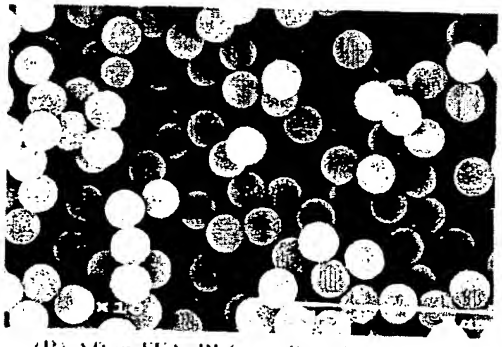

Intensity of FITC
250
200
150
100
50
0
0
1e+6
2e+6
3e+6
4e+6
Number of 1 μm FITC-, PS microspheres

FIG. 8

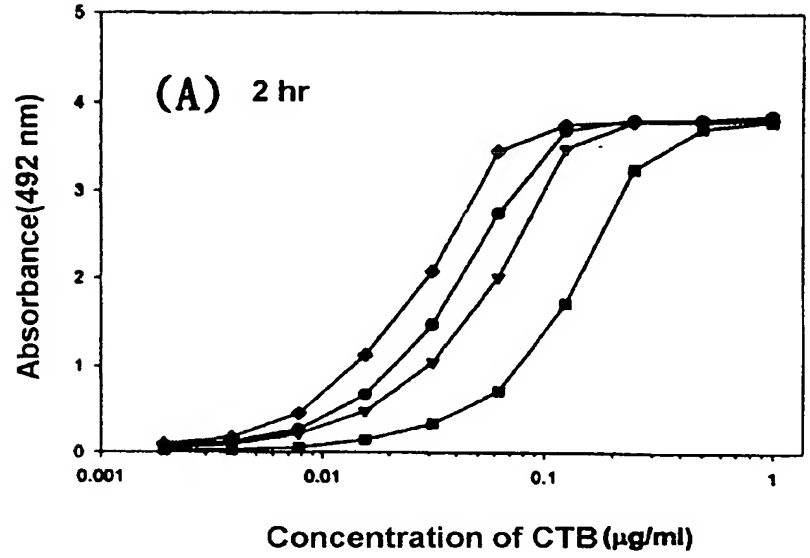


(A) Carboxylated PS microspheres



(B) After EDA, $PEG_{(5000)}$-PS microspheres

FIG. 9a


(A) 2 hr
Absorbance(492 nm)
0
1
2
3
4
5
0.001
0.01
0.1
1
Concentration of CTB (μg/ml)

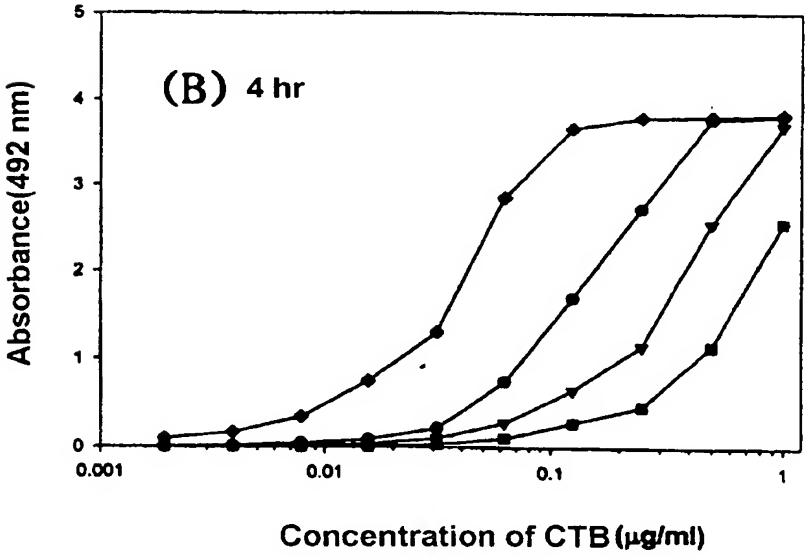
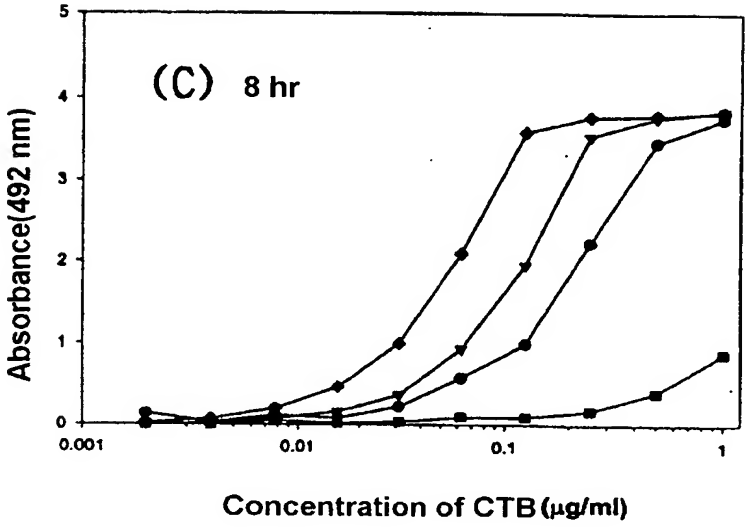

(B) 4 hr
Absorbance(492 nm)
0
1
2
3
4
5
0.001
0.01
0.1
1
Concentration of CTB (μg/ml)

FIG. 9b


(C) 8 hr
Absorbance(492 nm)
0
1
2
3
4
5
0.001
0.01
0.1
1
Concentration of CTB (μg/ml)

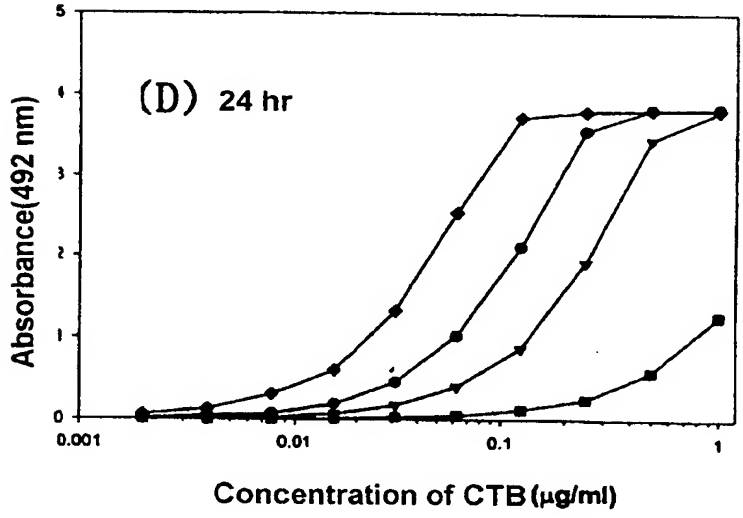

(D) 24 hr
Absorbance(492 nm)
0
1
2
3
4
5
0.001
0.01
0.1
1
Concentration of CTB (μg/ml)

FIG. 10

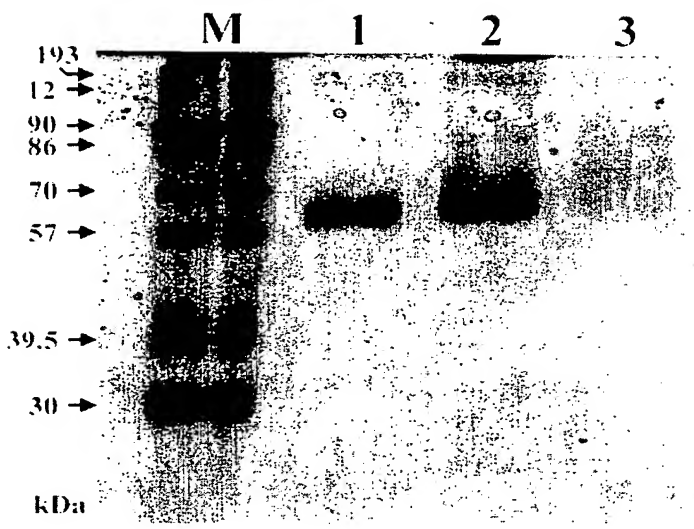


FIG. 11

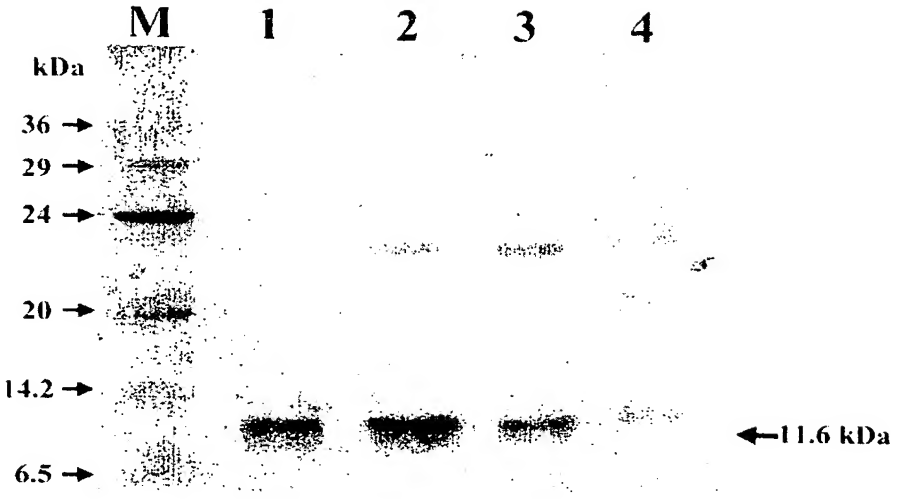
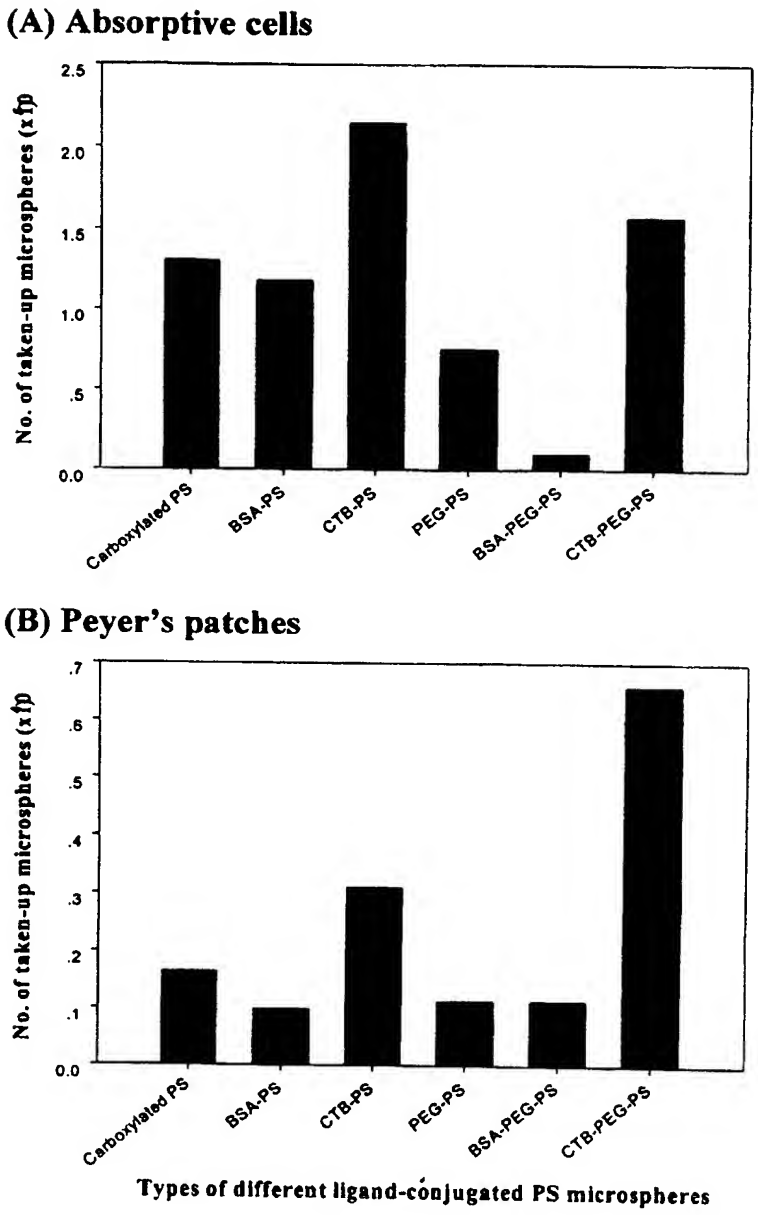


FIG. 12



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00534

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 9/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
IPC 7 : A61K

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
CA ON-Line

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98-33521 A1 (Korea Institute of Science and Technology) 06 August 1998 (06. 08. 98), see entire document	1- 14
Y	Almeida et al. 'Poly(lactic acid) microspheres as immunological adjuvants for orally delivered cholera toxin B subunit' In: Biochem. Soc. Trans., 1992, Volume 20, Number 4, page 316S, see entire document.	1- 14

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 AUGUST 2000 (30.08.2000)	31 AUGUST 2000 (31.08.2000)

Name and mailing address of the ISA/KR	Authorized officer
Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	YOON, Kyoung Aei Telephone No. 82-42-481-5609

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR00/00534

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 98-33521 A1	06. 08. 1998	AU 5681298 A1	25. 08. 98